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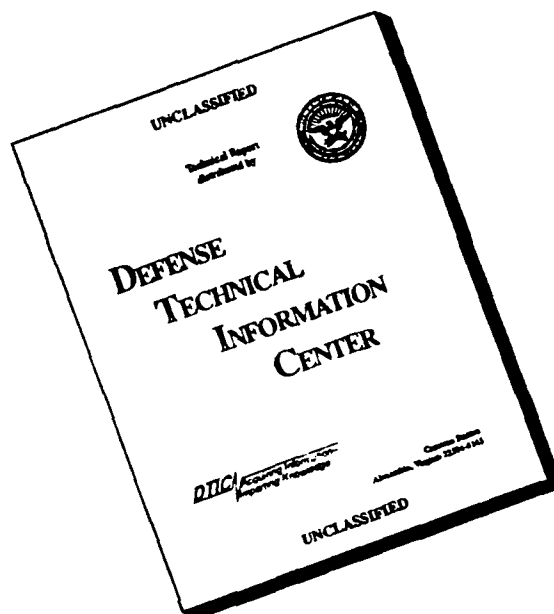
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Persistent Calcium Elevation Correlates with the Induction of Surface Immunoglobulin-mediated B Cell DNA Synthesis

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Summary

Surface immunoglobulin (sIg)-mediated stimulation of B lymphocytes induces a tyrosine kinase-dependent sequence of events leading to rapid and large elevations in intracellular ionized calcium ($[Ca^{2+}]_i$). These early biochemical events do not necessarily lead to proliferation of B cells, however, and conversely, the absence of or inhibition of these events does not necessarily prevent cellular proliferation. We now show by digital image analysis of single B cells that conditions which lead to B cell proliferation are associated with low-level but persistent sustained or cyclic elevations in $[Ca^{2+}]_i$. In marked contrast, early and nonsustained elevations in $[Ca^{2+}]_i$ are induced in B cells by stimuli that lead to G1 transition but fail to progress to DNA synthesis. Thus, when B cells were stimulated with mitogenic and nonmitogenic anti-IgD antibodies, both of which induce entry of cells into G1 and early calcium transients of comparable magnitude, persistent low-level calcium elevations were only detected in cells stimulated with the mitogenic antibody. Furthermore, persistent calcium elevations were also seen when B cells were stimulated with a multivalent dextran-anti-Ig conjugate which induced very high levels of B cell proliferation in the absence of detectable phosphatidylinositol 4,5-bisphosphate hydrolysis or elevations in $[Ca^{2+}]_i$ as detected by flow cytometry. Finally, B cells from X-linked B cell-defective mice, which do not proliferate in response to anti-Ig antibody, show marked and early increases in $[Ca^{2+}]_i$, but do not show persistent calcium elevations. These data suggest that the rapid and large increases of $[Ca^{2+}]_i$ seen in lymphocytes within seconds after antigen receptor ligation may be associated with entry in G1, whereas low-level but persistent elevations may be the hallmark of a cell destined to synthesize DNA.

Crosslinking of surface immunoglobulin (sIg)² receptors in B cells initiates a cascade of biochemical events that includes mobilization of intracellular ionized calcium $[Ca^{2+}]_i$ and activation of protein kinase C (PKC) and tyrosine kinases (1-7). Activation of PKC and tyrosine kinase in turn activates a number of other enzyme systems, including phosphatidylinositol 3 and mitogen-activated protein kinases, guanosine triphosphate-activating protein, and raf-1 protein

kinase (8-10), all of which are intimately involved in cell growth of B cells as well as other cell types. Although it is clear that the summation of these events reflects an early response to sIg-mediated signal transduction in B cells which will lead to cell growth, the specific role of each event in regulating entry of the cell into G1 or S of the cell cycle is not known. Experiments both in human and in murine systems suggest that there is no correlation between the ability of an antigen or of anti-Ig antibody to induce phosphatidylinositol 4,5-bisphosphate (PIP₂) activation or tyrosine kinase activation and its ability to induce B cell DNA synthesis (11-14). Thus, for example, inhibition of sIg-mediated PIP₂ hydrolysis does not prevent B cell proliferation (15). Furthermore, stimulation of these early events by anti-Ig antibodies does not necessarily lead to B cell proliferation (13, 16, 17).

¹ H. Yamada, C. H. June, and J. J. Mond contributed equally to the work.

² Abbreviations used in this paper: $[Ca^{2+}]_i$, intracellular free calcium concentration; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; sIg, surface Ig; TI, T cell-independent antigen.

An added level of complexity in understanding the role for these early biochemical events is created when trying to dissect differences between sIg-mediated events stimulated by T cell-dependent (i.e., mono or paucivalent) as compared with T cell-independent (multivalent and higher molecular weight) antigens (TI). Although both antigen types are similar in their abilities to induce the early events in B cell activation, T cell-dependent but not -independent, antigens require B cells to interact with T cells or T cell-derived cytokines to stimulate proliferation and Ig secretion.

We have recently described a model system utilizing dextran-conjugated anti-Ig antibodies which stimulates polyclonal activation of B cells via sIg ligation and crosslinking and mimics, in many respects, activation that is stimulated by TI type 2 antigens (18). These dextran conjugates stimulate high levels of B cell proliferation at picogram per milliliter concentrations that are not accompanied by detectable PIP_2 hydrolysis and tyrosine phosphorylation whereas higher concentrations of this ligand stimulate PIP_2 hydrolysis and tyrosine phosphorylation (11, 12, 16). Digital image analysis has made it possible to examine, at the level of the single B cell, some early activation events that may be masked when they are studied in cell populations (19). The experiments described in this manuscript demonstrate that sIg crosslinking-mediated induction of low-level and temporally persistent cyclic elevations in intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$) is a more critical determinant than the early induction of large increases in $[\text{Ca}^{2+}]_i$ for stimulating B cells to synthesize DNA.

Materials and Methods

Mice. DBA/2 mice (the Jackson Laboratory, Bar Harbor, ME) were used at 8 wk of age.

B Cell Purification. Suspensions of single spleen cells were washed

three times with RPMI 1640 (M.A. Bioproducts, Walkersville, MD) plus 10% FCS (GIBCO, Grand Island, NY) and treated with the following antibodies: monoclonal anti-Thy-1.2 ($1 \mu\text{g}/\text{ml}$) (20), anti-CD4 ($2.5 \mu\text{g}/\text{ml}$) (21), and anti-CD8 (1:400 dilution of culture supernatant) (20), respectively, per 10^7 spleen cells for 30 min on ice. This was followed by treatment with newborn rabbit complement (10%) (Pel-Freez Biologicals, Rogers, AR), in the presence of a 1:10 dilution of tissue culture fluid containing the anti-rat κ chain mAb, mouse anti-rat 18.5 (22), at 37°C for 45 min.

Cells were then fractionated into low- and high-density populations by Percoll gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, NJ; 3,000 rpm for 15 min). Gradients consisting of 70, 65, 60, and 50% Percoll (with densities of 1.086, 1.081, 1.074, and 1.062 g/ml, respectively) were used. The high-density cells (resting) were collected from the 70–65% interface. The average percentage of Ig^+ cells in the 70–65% fractions was 90–95%.

Fluorescence Digital Microscopy. Details of the image analysis system have been described (23, 24). Briefly, 5×10^6 cells were loaded with $1.5 \mu\text{M}$ fura-2 for 30 min at 31°C , washed, and suspended in HBSS with 0.1% FCS, 5×10^5 M 2-ME and 20 mM Hepes, pH 7.4. Cells were allowed to settle onto glass coverslips and equilibrated in a 2-ml microscope chamber at 37°C . Agonistic antibodies were gently added as a $10\times$ solution to the chamber. The fluorescence digital image processing system used is similar to that described previously by Tsien and Harootunian (23). Hardware consists of an image processor (model FD5000; Gould Inc., Glen Burnie, MD), a microscope (Axiovert; Zeiss, Oberkochen, Germany) with a 1.3 numerical aperture $\times 40$ objective, and a filter changer (Ludl Electronic Products, Hawthorne, NY) with excitation filters centered at 350 ± 10 nm and 380 ± 10 nm. The image processor and filter changer are interfaced to a host computer (Microvax; Digital Equipment Corp., Marlboro, MA). 16 images were acquired at each wavelength at 30/s through a camera (CCD; Cohu Inc., San Diego, CA) and image intensifier (Video Scope Intl., Ltd., Wash, DC). The images were averaged, background subtracted, and a shading correction applied. The ratio of the 350- and 380-nm images is displayed according to a code kindly provided by R. Y. Tsien (University of California, San Diego, CA). Images

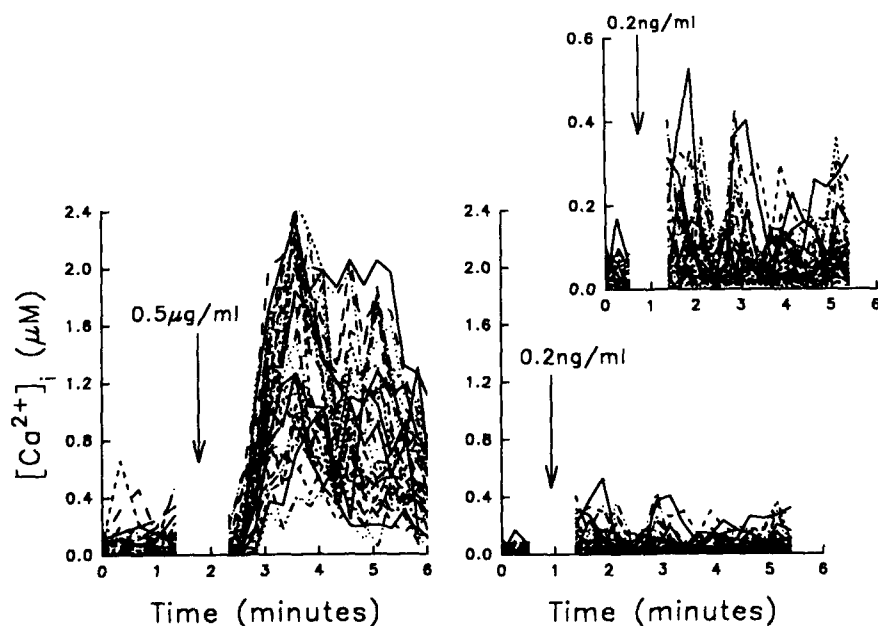


Figure 1. Fura-2 B cells were settled on coverslips and stimulated with $0.5 \mu\text{g}/\text{ml}$ or $0.2 \text{ ng}/\text{ml}$ of $\text{H}\delta 1/-$ dextran. Results show the $[\text{Ca}^{2+}]_i$ of individual B cells for the first 6 min after stimulation; there were 35 and 36 cells in the left and right panels, respectively. Each line represents the $[\text{Ca}^{2+}]_i$ of an individual cell analyzed over 6 min. This experiment is one of three representative experiments.

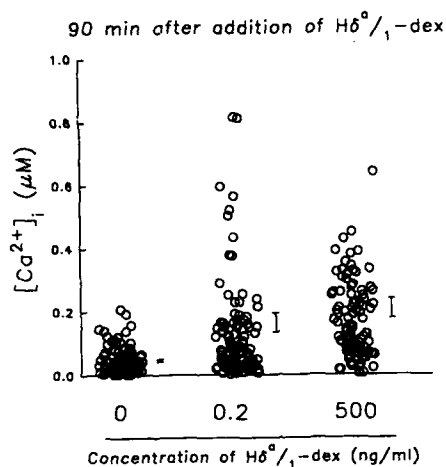


Figure 2. B cells were treated as in Fig. 1 and evaluated by image analysis at 90 min after the addition of $H\delta^1/1$ -dextran. (Circles) The $[Ca^{2+}]_i$ of an individual cell at 90 min; 105–120 cells per condition are plotted. Bars represent the mean $[Ca^{2+}]_i \pm SEM$. This experiment is one of four representative experiments.

were acquired and stored digitally at 15-s intervals. The fura-2 ratio is converted to $[Ca^{2+}]_i$ as described (23).

Representation of Data. The error bars in the graphs represent the SE of the mean $[Ca^{2+}]_i$ of individual cells.

Results

Short-Term Calcium Transients Stimulated by Picogram Quantities of Anti-Ig Antibody. We have previously reported that picogram per milliliter concentrations of dextran-conjugated anti-Ig antibody (anti- δ -dex), which stimulate high levels of B cell DNA synthesis, do not stimulate detectable increases

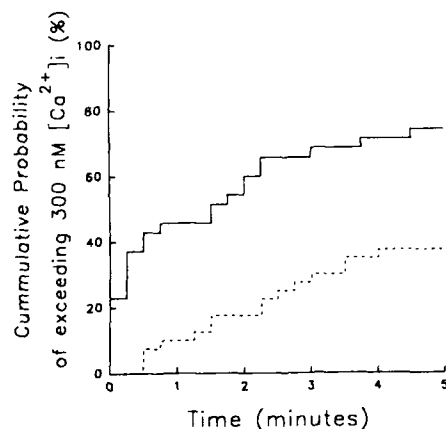


Figure 3. Frequency of responding cells late after stimulation. Data from the experiment shown in Fig. 2 were analyzed for the cumulative frequency of responding cells. Cells in one microscopic field were analyzed beginning 90 min after stimulation for a further 5 min. Cells were considered as responding cells if the $[Ca^{2+}]_i$ exceeded 0.3 μM . Cells were cultured in medium (·····), $H\delta^1/1$, 0.2 ng/ml (----), or $H\delta^1/1$, 0.5 $\mu g/ml$ (—). The total number of cells per field for the three conditions was 35, 40, and 35, respectively.

in $[Ca^{2+}]_i$ as measured by flow cytometry (11). In contrast, microgram per milliliter concentrations of this antibody retained mitogenicity and stimulated early onset $[Ca^{2+}]_i$ elevations that were high in magnitude. To evaluate whether analysis of a population of cells by standard flow cytometric approaches might be masking small but significant perturbations of $[Ca^{2+}]_i$ after B cell activation, we studied sIg-mediated B cell activation by digital image analysis. This technique allows one to study events of single cells over real time and to detect oscillatory responses that may not be apparent by flow cytometry (23, 24). Purified resting B cells were allowed to settle for 5 min onto coverslips and then placed in thermostatically controlled chambers at 37°C. Anti- δ -dex at 0.5 $\mu g/ml$ stimulated rapid increases in $[Ca^{2+}]_i$ in >90% of the B cells to levels that were, on average, 8–10-fold greater than those of unstimulated B cells (Fig. 1, left). This result is similar to that which we reported previously using flow cytometric analysis of indo-1-loaded B cells (11). As has previously been described, B cell $[Ca^{2+}]_i$ levels showed either sustained or oscillatory patterns of calcium elevation (19). The magnitude of the response of each cell and its oscillatory frequency exhibited substantial heterogeneity for the ~200 cells examined. When B cells were stimulated with highly mitogenic but 1,000-fold lower concentrations of anti- δ -dex (0.2

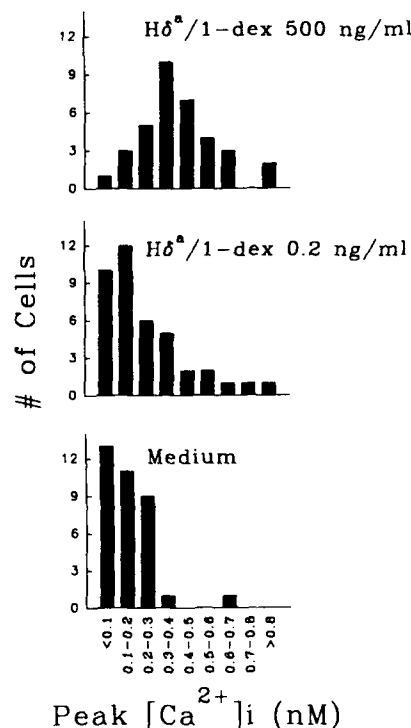
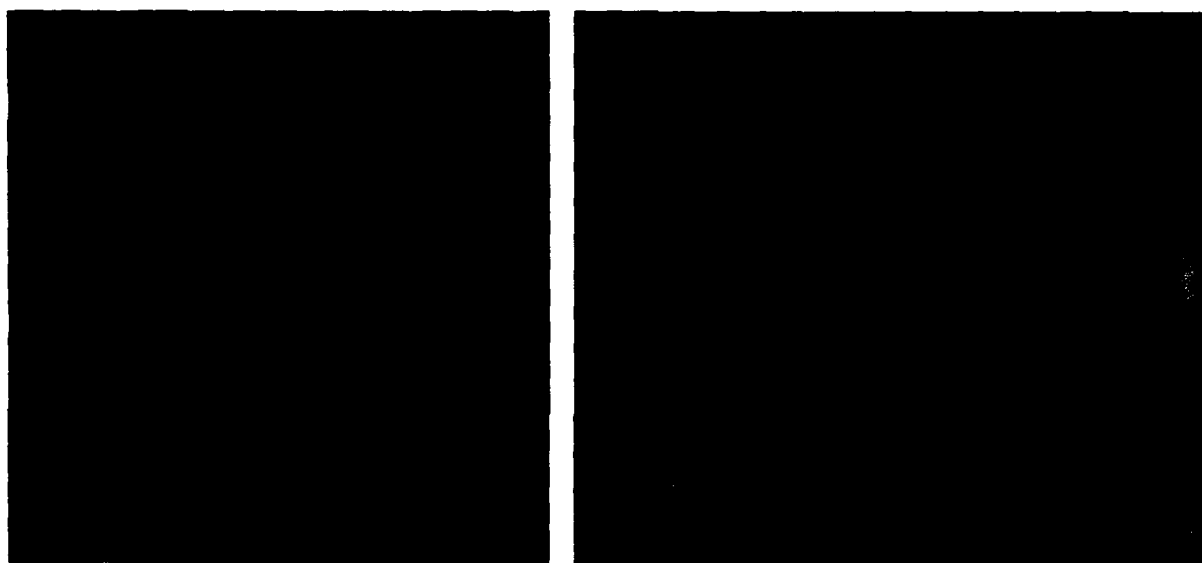
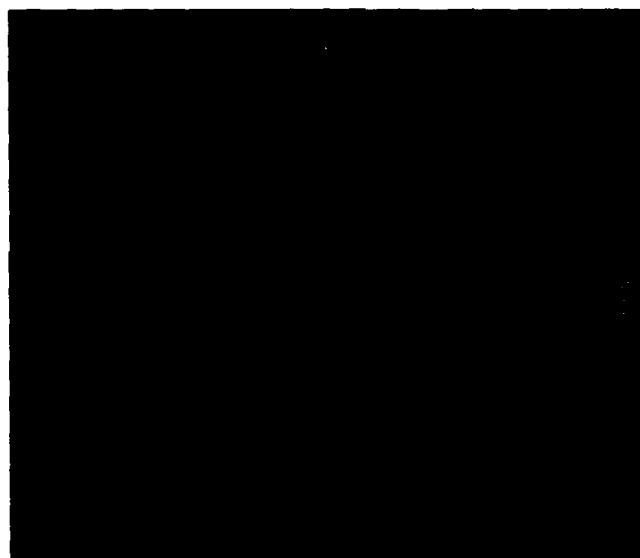


Figure 4. Histograms of peak $[Ca^{2+}]_i$ late after stimulation. Cells from one microscopic field from the experiment shown in Figs. 2 and 3 were analyzed 90 min after stimulation for a further 5 min. Data were obtained at 15-s intervals, and histograms of the peak $[Ca^{2+}]_i$ for each cell are plotted. Cells were cultured in medium, $H\delta^1/1$, 0.2 ng/ml, or $H\delta^1/1$, 500 ng/ml. The total number of cells per field for the three conditions were 35, 40, and 35, respectively.



DBA/2 60 min after H δ a /1

DBA/2 60 min after FF1



DBA/2 60 min in medium

Figure 5. B cells were stimulated with the mitogenic H δ /1 anti- δ antibody or the nonmitogenic FF1 anti- δ antibody. $[Ca^{2+}]_i$ was evaluated immediately after the addition of anti-Ig antibody (three pseudo-color photos, *right*) or 60 min after the addition of antibody (three pseudo-color photos, *left*). Chambers were kept in an incubator at 37°C, 5% CO₂ for the interim 60 min. Because of the necessity of maintaining cells in an incubator for this prolonged period of time, a different field of cells was analyzed at 0 time and at 60 min. However, for each time point, three separate fields of at least 30 cells/field were analyzed. Results are depicted for one representative field. The color legend (*right*) reflects the $[Ca^{2+}]_i$ of each cell, with blue being the lowest and violet the highest $[Ca^{2+}]_i$. The halo at the periphery of some cells is an artifact and does not indicate a spatial intracellular $[Ca^{2+}]_i$ gradient.

ng/ml), smaller increases in $[Ca^{2+}]_i$ were also consistently observed (Fig. 1, *right*). In our previous flow cytometric studies, we were unable to detect a response in B cells stimulated with this low dose of anti- δ -dex (11). The response detected by digital microscopy was more apparent when the results were depicted using a smaller scale (Fig. 1, *inset*). Whereas, at any given time, <25% of B cells stimulated with low concentrations of anti- δ -dex showed increases in $[Ca^{2+}]_i$, >90% of B

cells showed significant responses in the five min after stimulation. Although the amplitude of the oscillations under these conditions was small, the frequency was not appreciably different than that stimulated by the higher concentrations of anti- δ -dex. This suggested that the persistence of oscillations may be a more critical factor than the magnitude of initial calcium elevation in determining the calcium-dependent events that lead to B cell activation.

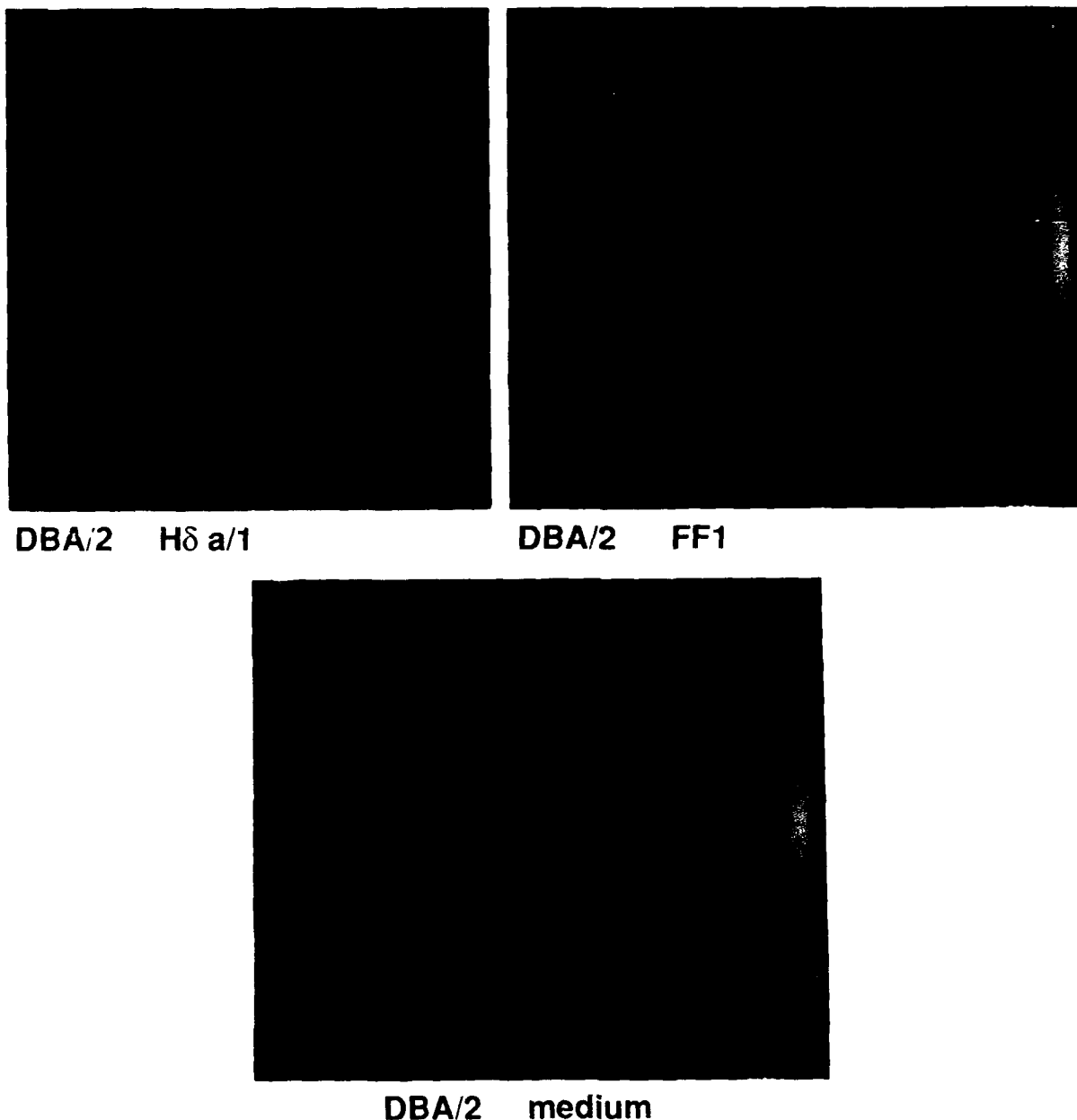


Figure 5. (continued)

Prolonged Calcium Oscillations Stimulated by Mitogenic but Not by Nonmitogenic Concentrations of Anti- δ Antibodies. The reports that B cell proliferation stimulated by anti-Ig antibody appears to require prolonged exposure to antigen or anti-Ig antibody suggested that persistent signalling was a critical factor in directing a B cell to DNA synthesis (25). To study this point directly, we evaluated calcium transients at both early and late time points after B cell stimulation by mitogenic amounts of anti- δ -dex antibody at high (500 ng/ml) or low (0.2 ng/ml) concentrations. By 90 min after activation, the mean increase in $[Ca^{2+}]_i$ stimulated by the high concentration of anti- δ -dex had fallen significantly from

its peak level seen seconds after stimulation, whereas that stimulated by the low concentration stayed relatively constant over the 90 min of observation (Fig. 2). In six experiments of this design, the mean increase in $[Ca^{2+}]_i$ in cells 90 min after stimulation with 500 ng/ml was either the same as, or only marginally greater than that stimulated by 0.2 ng/ml.

The above results were obtained by observing three microscopic fields for each condition at one time point 90 min after stimulation. It was necessary to sum the results from several fields of cells for statistical purposes. However, observation of the same field of cells as a function of time at 15-s

intervals supplies additional information, and reveals that the response pattern of individual cells is dynamic, and consists of cells with both sustained elevations of $[Ca^{2+}]_i$ and cells with oscillatory elevations (19). When the response of a particular field of cells was analyzed as a function of time, observations were necessarily limited to the number of cells contained in one field of cells. Typically, there are 30–50 cells. In one experiment, it was found at the first observation point that there were 23% of cells with increased $[Ca^{2+}]_i$ in the group cultured for 90 min in $H\delta^a/1$ -dex at 0.5 $\mu\text{g}/\text{ml}$, whereas there were no cells with elevated $[Ca^{2+}]_i$ in the groups cultured in low-dose $H\delta^a/1$ -dex 0.2 ng/ml or in medium. However, over the next 5 min, there was a progressive increase in the fraction of cells that had elevated $[Ca^{2+}]_i$, reaching 69 and 38% in the high- and low-dose $H\delta^a/1$ conditions, whereas only 2 of 35 cells (6%) of cells cultured in medium had elevated $[Ca^{2+}]_i$ (Fig. 3). Histograms of these responses showed that the magnitude of peak calcium increases in the time period of 90–95 min after stimulation was similar in the low- and high-dose $H\delta^a/1$ -dex groups (Fig. 4). Thus, the magnitude of calcium elevations late after stimulation appears to be similar in both the high- and low-dose $H\delta^a/1$ -dex conditions. We are unable to determine if the frequency of responding cells differs between the two groups. During the 5 min of analysis, the high-dose condition appears to approach a plateau, whereas the plot of low-dose condition suggests that a higher fraction of responding cells might be observed with longer periods of observation (Fig. 3). Currently, we are unable to distinguish between these possibilities because of leakage of the calcium probe with prolonged cell culture. These results indicate that high-dose $H\delta^a/1$ causes large and early transient increases in B cell calcium whereas low-dose $H\delta^a/1$ results in only minimal early increases in calcium that are detectable by video microscopy (Fig. 1) and not by flow cytometry (11). In contrast, when B cells are examined 60–90 min after stimulation, both doses of $H\delta^a/1$ result in calcium elevations in a substantial fraction of cells (Fig. 3), of which the magnitude of the elevations is similar (Figs. 2 and 4). Together, these results suggested that persistent and prolonged signalling, as reflected by low-level calcium transients, was a critical element in determining whether a cell would proceed to mitogenesis. Alternatively, this form of signal transduction might serve as a marker of cells that are committed to mitogenesis.

To further test this, we compared the ability of a mitogenic ($H\delta^a/1$) and a nonmitogenic (FF1-4D5) monoclonal anti- δ antibody (26) to stimulate immediate as well as prolonged elevations in $[Ca^{2+}]_i$. Unconjugated $H\delta^a/1$, in contrast to the dextran conjugate, is mitogenic over a narrower concentration range of 1–100 $\mu\text{g}/\text{ml}$ (11, 18). Both $H\delta^a/1$ and FF1-4D5 stimulated comparable increases in $[Ca^{2+}]_i$ within seconds after stimulation (Fig. 5). In marked contrast, by 60 min after stimulation, cells stimulated by the mitogenic, but not the nonmitogenic, anti- δ antibody showed small but significant elevations in calcium (Figs. 5–7). These prolonged cyclic elevations were not seen when nonmitogenic concentrations of $H\delta^a/1$ (0.02 $\mu\text{g}/\text{ml}$) were employed (Fig. 7). When B cells from C57BL/6 mice, which express Ig of

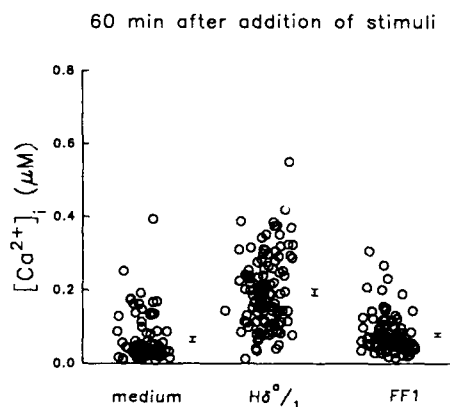


Figure 6. Experimental design same as in Fig. 5, with results depicted as a scattergram. (Circles) The $[Ca^{2+}]_i$ of one cell at 60 min after stimulation. The bars represent mean $[Ca^{2+}]_i \pm \text{SEM}$. In the medium, $H\delta^a/1$, and FF1 cultures, there were 80, 120, and 100 cells, respectively.

the b allotype and thus are nonreactive to $H\delta^a/1$ (anti-allotype IgD^a) were examined, no short- or long-term increase in calcium transients was observed, indicating that these low-level oscillations are not attributable to artifacts of the in vitro culture system (Fig. 8). These results suggest that persistent but low-level elevations in $[Ca^{2+}]_i$ are associated with stimulation of B cells by mitogenic but not nonmitogenic anti-Ig antibodies.

Absence of Long-Term Calcium Transients in Anti-Ig-stimulated B Cells from *xid* Mice. CBA/N mice have been shown to have an X-linked immune defect (*xid*) and do not respond to immunization with TI type 2 antigens (27). Furthermore, whereas unconjugated anti-Ig antibody does not stimulate proliferation in B cells from *xid* mice, dextran-conjugated anti-Ig antibody stimulates high levels of proliferation (13). In view of this finding, we wished to test whether dextran-conjugated antibody, but not unconjugated anti-Ig, would stimulate long-term calcium transients in B cells from *xid*

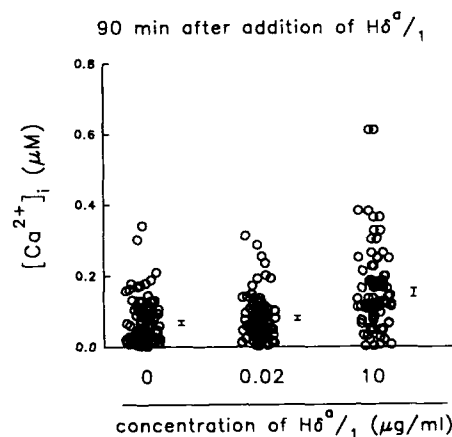


Figure 7. Fura-loaded B cells were cultured in medium (0), or stimulated with a mitogenic (10 $\mu\text{g}/\text{ml}$) or nonmitogenic (0.02 $\mu\text{g}/\text{ml}$) concentration of unconjugated $H\delta^a/1$ and analyzed at 90 min. In the 0, 0.02, and 10 $\mu\text{g}/\text{ml}$ conditions, there were 100, 90, and 90 cells, respectively. This experiment was one of three representative experiments.

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